Relationship between expression of sex steroid receptors and structure of the seminal vesicles after neonatal treatment of rats with potent or weak estrogens

Relationship between Expression of Sex Steroid Receptors and Structure of the Seminal Vesicles after Neonatal Treatment of Rats with Potent or Weak Estrogens

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In this study we evaluated the effect of manipulating the estrogen and androgen environment of the neonatal male rat on subsequent immunoexpression of sex steroid receptors in the seminal vesicles (SVs) at age 18 days. The aim was to establish to what extent such changes were associated with and predictive of changes in SV structure/composition. Treatments were either diethylstilbestrol (DES; 10, 1, or 0.1 μg/injection), ethinyl estradiol (EE; 10 μg/injection), tamoxifen (2 mg/kg/day), flutamide (50 mg/kg), a gonadotropin-releasing hormone antagonist (GnRHa; 10 mg/kg), genistein (4 mg/kg/day), octylphenol (2 mg/injection), or bisphenol A (0.5 mg/injection). Compared with controls, treatment with DES (10 μg) induced loss of epithelial and stromal androgen receptor (AR) immunoexpression coincident with induction of stromal progesterone receptor (PR) immunoexpression and upregulation of stromal immunoexpression of estrogen receptor-α (ERα). These changes were associated with gross distortion (increase) of the normal stromal:epithelial tissue proportions in the SVs. DES (1 μg) and EE induced similar but less pronounced changes, and DES (0.1 μg) had no noticeable effect. Tamoxifen and flutamide induced PR and slightly upregulated ERα immunoexpression but had only a minor or no effect on AR expression and the stromal:epithelial ratio, though flutamide retarded normal development of the SVs. The latter was also evident in GnRHa-treated males, but otherwise this treatment had no effect on AR and PR immunoexpression. None of the foregoing treatments had any detectable effect on the immunoexpression of ERβ in stromal or epithelial cells. The major treatment-induced changes in immunoexpression of AR, PR, and ERα and lack of change in ERβ were confirmed by Western blots of SV protein extracts. None of the three weak (environmental) estrogens tested caused any detectable change in sex steroid receptor immunoexpression or SV tissue composition. We conclude that treatment-induced loss of AR is a prerequisite for altered stromal:epithelial proportions in the SVs and that such loss is always associated with induction of PR and upregulation of ERα; the latter two changes are insufficient on their own to bring about such a change. Nevertheless, induction of PR expression was always associated with altered SV development and is a potentially useful marker because it is not normally expressed in male reproductive tissues. *Key words:* **androgen–estrogen balance, androgen receptor, epithelium, estrogen receptor-α, estrogen receptor-β, progesterone receptor, stroma.** *Environ Health Perspect* **109:1227–1235 (2001). [Online 24 November 2001]**
http://ehpnet1.niehs.nih.gov/docs/2001/109p1227-1235williams/abstract.html

Ever since the work of Jost (*1,2*), it has been recognized that hormones, particularly testicular androgens, play a vital role in the development of the male reproductive system. More recent work, using approaches such as the administration of antiandrogens (e.g., flutamide) or inhibitors of 5α-reductase (e.g., finasteride), has confirmed this role (*3*). Similarly, the widespread expression of androgen receptors (AR) in stromal and epithelial cells throughout the reproductive tract of the male during fetal and neonatal life (*4,5*) and the failure of these tissues to develop normally when inactivating mutations of the AR are present (*6,7*) also point to important effects of androgens on these target cells. However, in the last few years it has become apparent that estrogen receptors (ER), predominantly ERβ, are distributed as widely as are AR in male reproductive tissues during development (*8–11*), and coexpression of AR and ERβ and/or ERα in stromal and epithelial cells probably occurs throughout most of the reproductive tract (*11–13*) Although knockout studies involving ERα and/or ERβ have failed so far to identify major changes in the developing reproductive tract (*12*), with the notable exception of the efferent ducts in male ERα knockout mice (*14*), studies in which rodents have been exposed to exogenous estrogens administered during pregnancy or to pups neonatally have shown major adverse effects on all parts of the developing reproductive tract (*15,16*). This includes effects on the testis (*11,17*), efferent ducts (*18,19*), epididymis and vas deferens (*10,11*), prostate (*20,21*), and seminal vesicles (*9*). Such findings have helped fuel concern about potentially similar adverse effects of environmental estrogens on the developing male.

The contrast between absence of major reproductive tract abnormalities in transgenic mice in which estrogen action is restricted and the widespread adverse changes that occur after administration of exogenous estrogens to normal rats and mice is puzzling. Our recent studies have suggested possible explanations for this puzzle. First, we have shown that doses of estrogens that cause abnormalities of the testis, efferent ducts, epididymis, and vas deferens in rats when administered neonatally also induce widespread loss of expression of AR (*11*), thus restricting the ablity of androgens to act on these tissues; similar changes have been reported in earlier studies of the developing prostate (*22,23*). Second, we have shown that neonatal estrogen treatment results in dose-dependent induction of progesterone receptor (PR) expression in stromal, but not epithelial, cells throughout much of the reproductive tract of the male (*9*). Estrogen induction of PR expression is recognized as one of the classic effects of estrogen action on the uterus (*24*), but in normal male rats PR expression in reproductive tract tissues is completely absent (*9*). Studies by others have also shown that estrogens can induce PR expression in male reproductive tissues (*25–27*) as well as expression of lactoferrin in the seminal vesicles (*28–31*)—lactoferrin also being a uterine protein that is inducible by estrogens. Estrogen induction of PR and lactoferrin in male reproductive tract tissues is enhanced by castration (*25,28*) (i.e., by the removal of androgens). This implies that the balance in action between androgens and estrogens may be a critical factor in determining the response of the developing reproductive tract to exogenous estrogens. We reached a similar conclusion in our own studies, but also suggested that induction of abnormalities occurred only when androgen action was impaired coincident with supranormal estrogen action (*11*). Taken together, these findings suggest that altered expression of AR (reduced) and/or PR (induced) may be defining features of estrogen induction of abnormalities of the developing male reproductive

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We are grateful to R. Deghenghi and Europeptides for their generous gift of GnRH antagonist and to J. MacDonald for expert assistance.

This work was supported in part by the European Centre for the Ecotoxicology of Chemicals (ECETOC), by a Strategic Research Fund Award from AstraZeneca plc and by contract QLK4-1999-01422 from the European Union.

Received 5 February 2001; accepted 2 May 2001.

system. Other evidence suggests that altered expression of ERs, particularly increased expression of ERα, could also be important in this regard (*10,32–34*).

Although the findings outlined above are strongly suggestive, there are important data gaps and some inconsistencies. For example, it is unclear whether interference with androgen production or action alone during development (i.e., in the absence of estrogen treatment) induces changes in sex steroid receptor expression. It is also unresolved whether changes induced by estrogens are also evident at low doses and whether weak environmental estrogens might also induce comparable changes. To address these issues, we sought to identify which manipulations of the sex steroid environment in neonatal life in the rat were able to alter expression of AR, PR, and ERs in a target tissue of the developing male reproductive tract. For this purpose we chose the seminal vesicles because our preliminary findings and others in the literature (see above) had suggested that estrogen-induced changes in receptor expression were clearly demonstrable in this tissue.

Materials and Methods

Animals and treatments. Wistar rats were bred and housed in our animal facility under standardized conditions. On day 2 (day of birth = day 1), all-male litters of 8–14 pups were derived by cross-fostering, and one or other of the following treatments was initiated by subcutaneous injection.

a) We administered diethylstilbestrol (DES; Sigma, St. Louis, MO, USA) at a dose of either 10, 1, or 0.1 μg in 20 μL corn oil on days 2, 4, 6, 8, 10, and 12. We have shown previously that this dosing regime encompasses doses that do (10 μg) or do not (0.1 μg) induce reproductive tract abnormalities and changes in AR and PR expression (*9,11,35*).

b) We administered ethinyl estradiol (EE; Sigma) similarly to DES at a dose of 10 μg per injection.

c) We administered the mixed estrogen agonist/antagonist tamoxifen (Sigma) at a dose of 2 mg/kg in 20 μL corn oil on days 2–16. This dose was chosen based on previous studies in the literature (*36*).

d) We administered bisphenol A (BisA; Aldrich Chemicals Limited, Dorset UK) at a dose of 0.5 mg in 20 μL corn oil on days 2–12. This was the highest dose that could be kept in solution for administration and is capable of inducing biologic effects on the developing male (*35*).

e) We administered 4-*tert*-octylphenol (Aldrich) at a dose of 2 mg in 20 μL corn oil on days 2–12. This was the highest dose that could be kept in solution for administration and is capable of inducing biologic effects on the developing male (*35*).

f) We administered genistein (Sigma), an isoflavenoid phytoestrogen, at a dose of 4 mg/kg/day in 2 mL/kg vehicle, as described elsewhere (*35*). This dose was chosen based on reported values for total isoflavenoid intake by human infants fed on a 100% soy formula diet (*37*).

g) We administered a potent, long-acting GnRH antagonist (GnRHa; Antarelix, Europeptides, Argenteuil, France) at a dose of 10 mg/kg in 20 μL 5% mannitol on postnatal days 2 and 5 only. We have shown that this treatment regime is sufficient to switch off pituitary gondotropin secretion until beyond day 18 with resultant suppression of Leydig cell development and of endogenous testosterone levels (*11,13,17*). This suppression causes retardation of development of the testis similar to that observed in DES (10 μg)-treated animals and helps distinguish whether DES-induced changes stem from this change rather than a specific effect of estrogen action.

h) We administered the AR antagonist flutamide (Sigma) at a dose of 50 mg/kg in 20 μL corn oil on days 2, 4, 6, 8, 10, and 12. This dose was chosen because it interferes with masculinization of male pups when administered to pregnant rats (*3*).

i) We administered the appropriate vehicle for the appropriate period (e.g., 20 μL corn oil on days 2, 4, 6, 8, 10, and 12 as a control). Because no discernible differences were apparent among the various control groups used for treatments, data from these animals were pooled for analysis.

Tissue recovery, fixation, and processing. Animals were killed on postnatal day 18 by inhalation of either flurothane or CO_2 followed by cervical dislocation. Day 18 was chosen for study because our previous studies have shown that at this age there are maximal changes in AR and PR expression after estrogen treatment (*9–11*). We collected tissue from groups of 11–15 animals from each of the treatment groups specified above, except for treatment *c*, where $n = 3$. The bladder–prostate–seminal vesicle complex was dissected out whole and was immersion fixed in Bouin's fixative for 5.5 hr at room temperature. At this point the seminal vesicles were dissected away from the bladder complex and transferred into 70% ethanol before being processed into paraffin blocks in an automated processor. In some instances, seminal vesicles were dissected free at the time of death and were frozen for protein extraction as described below.

Antibodies. For immunolocalization studies we used the following antisera: ERβ was immunolocalized with a polyclonal antibody raised in sheep (S40) to a peptide specific for the hinge (D) domain of human ERβ conjugated to keyhole limpet hemocyanin (Affinity, Exeter, UK) according to standard methods (Diagnostic Services Scotland, Carluke, UK) as previously described (*9*). Immunolocalization of ERα used monoclonal antibody NCL-ER-6F11 produced by NovoCastra (Newcastle upon Tyne, UK) (*9,10*). PR was immunolocalized using a polyclonal C-19 antibody produced by Santa Cruz as described previously (*9*). AR was immunolocalized using the polyclonal N-20 antibody from Santa Cruz (*11*). The specificity of the various antibodies has been demonstrated in our laboratory by using Western blots (see also below), and further details can be found in the references cited.

Immunohistochemistry. Sections were cut at 3–5 μm and floated onto slides coated with 2% 3-aminopropyltriethoxy-silane (Sigma) and dried overnight at 50°C. Slides were dewaxed and rehydrated, and endogenous peroxidase was blocked using 3% (v/v) hydrogen peroxide in methanol. After washing in water, sections underwent antigen retrieval by pressure cooking at 1 bar/5 min in 0.01 M citrate buffer, pH 6 (PR, AR, and ERα) or in 0.01 M glycine 0.03% EDTA, pH 3.5 (ERβ). After release of the pressure valve, the sections were allowed to stand for 20 min, washed twice (5 min each) in 0.05M Tris-buffered saline (TBS), pH 7.4, and blocked for 30 min with 20% normal rabbit serum (NRS) for ERα and ERβ or with normal swine serum (NSS) containing 5% bovine serum albumin for AR and PR. The antiserum/preabsorbed antibody (see below) was then diluted in the appropriate normal serum (ERα 1:20 dilution NRS, ERβ 1:1,000 dilution NRS, PR and AR 1:200 NSS) and 100–200 μL was added to each slide before incubation at 4°C overnight in a humidity chamber. The slides were then washed in TBS (2 × 5 min) before incubation for 30 min with a biotinylated second antibody—namely, rabbit antimouse (ERα) or swine antirabbit (AR, PR; all from Dako, High Wycombe, Buckinghamshire, UK) or rabbit antisheep serum (ERβ; Vector Labs, Burlinghame, CA, USA), diluted 1:500 in 20% normal serum. After two washes in TBS (2 × 5 min), avidin–biotin-conjugated horseradish peroxidase (Dako) was applied for 30 min. After two final washes in TBS, a solution of diaminobenzidine was applied (Dako). The slides were developed until the color reached the required intensity in control sections, and the reaction was then stopped by immersing the slides in distilled water. Slides were counterstained with hematoxylin before being dehydrated by immersion in a graded series of ethanols and then being cleared in xylene. A coverslip was fixed over the sections using Pertex mounting medium (Cell Path, Hemel Hempstead, UK).

To confirm the specificity of the various antibodies used for immunocytochemistry, the primary antibody was preabsorbed with 10 × w/w peptide/recombinant protein to which the antibody was raised (PR, ERβ, and ERα) or by omission of the primary antibody (AR). In each case, this abolished all immunostaining for each of the antibodies used in this study. Full details of these procedures can be found elsewhere (*9–11,38*).

Evaluation of immunoexpression and its semiquantitation. To ensure the reproducibility of findings, we repeated immunolocalization studies for PR, AR, ERβ, and ERα on three to five separate occasions using sections from at least three animals in each of the treatment groups. Sections from animals in each of the treatment groups were run in parallel. We used a subjective scoring method (ranging from – to +++) to score the intensity of immunostaining for each of the receptors in stromal and epithelial cells of the seminal vesicles. For this evaluation, the scoring was made by reference to tissues from control and rats treated with 10 µg DES. The latter group was chosen as a positive treatment control in which major changes in immunoexpression of AR, PR, and ERα were induced. To aid further in the semiquantitation, we included tissues from animals treated with lower doses of DES (1 or 0.1 µg) to provide a reference dose–response curve. The average scores for intensity of immunoexpression are based on systematic analysis of a total of at least six animals from two separate experiments, except for tamoxifen-treated animals; for these, the data are based on three animals from one experiment.

We examined and photographed immunostained sections using an Olympus Provis microscope (Olympus Optical, London, UK) fitted with a Kodak DCS330 camera (Eastman Kodak, Rochester, NY, USA). Captured images were stored on a Macintosh G4 computer (Apple Computer, Inc., Cupertino, CA, USA) and compiled using Photoshop 5.0 (Adobe Systems, Inc., Mountain View, CA, USA) before being printed using an Epson Stylus 750 color printer (Seiko Epson Corp., Nagano, Japan).

Protein extracts and Western analysis. Seminal vesicles (SV) from some of the animals described under treatments were dissected out immediately after death and frozen on dry ice. These tissues were stored at –70°C until required. Whole SVs were then ground to a powder under N_2 in a pestle and mortar and transferred into tubes cooled on dry ice. Approximately 200 mg of ground tissue was transferred onto ice and 200–400 µL cold buffer A [1 mM Hepes, pH 7.9; 10 mM KCl; 0.1 mM EDTA; 0.1 mM EGTA; 1 mM dithiothreitol; 1x protease complete (Roche, Lewes, East Sussex, UK)] was added and the tube vortexed. The tissue was incubated at 4°C for 15 min to allow the cellular membranes to swell before the addition of 25 µL 10% Nonidet NP-40 (Sigma) and vigorous vortexing of the tube. The tissue homogenate was then microcentrifuged for 30 sec at 4°C before determination of protein concentration by spectrophotometry. The supernatant was then snap frozen in 100- and 400-µg aliquots that were discarded after a single thaw cycle.

Protein samples were analyzed on 4–20% gradient SDS-PAGE gels (Invitrogen, Groningen, the Netherlands) used according to the manufacturer's instructions. The gels were loaded with the denatured protein sample and were run in parallel with prestained molecular weight markers (Bio-Rad Laboratories, Hertfordshire, UK). The gels were then transferred onto polyvinylidene difluoride membranes (Immobilon-P; Millipore, Watford, UK) using a Novex miniblotter and following the manufacturer's instructions. The membranes were then blocked for 2–3 hr in 5% skimmed milk powder (Marvel) in TBST (TBS, 0.05% Tween-20; Sigma). The PR antibody was added at a dilution of 1:200, ERα antibody at 1:50, AR antibody at 1:200, and ERβ antibody at 1:2,000 and incubated overnight at 4°C. After repeated washing (2 × 15 min then 4 × 5 min) in TBST, the relevant peroxidase-conjugated second antibody was added (1:4,000) in 5% skimmed milk/TBST, and incubation continued for 2 hr. The second antibodies used were donkey antirabbit (Amersham), donkey antimouse (Amersham), or rabbit antisheep (Diagnostic Services Scotland, Carluke, UK). After repeated extensive washes (2 × 15 min then 4 × 5 min), specific signals were detected using the ECL detection system (Amersham) and hyperfilm (Kodak) following the manufacturers' instructions.

Preabsorption with 10 × w/w peptide/recombinant protein to which the antibody was raised (PR, ERβ, and ERα) abolished all reactivity on Western blots for each of the antibodies used in this study (*9,10*).

Table 1. Summary of gross morphologic changes to the seminal vesicles in the various treatment groups.

Neonatal treatment	Stromal:epithelial ratio	Epithelial branching
DES 10 µg	Increased +++	Reduced +++
DES 1 µg	Increased +	Reduced +
DES 0.1 µg	Normal	Normal
EE 10 µg	Increased +	Reduced +
Tamoxifen	Increased ±	Reduced +
Bisphenol A	Normal	Normal
Octylphenol	Normal	Normal
Genistein	Normal	Normal
GnRH antagonist	Normal	Normal
Flutamide	Normal	Reduced +

The magnitude of any change is scored as + to +++ and is based on analysis of at least six animals per group (except for tamoxifen, where *n* =3).

Table 2. Summary of changes in the intensity of immunoexpression of sex steroid receptors in the seminal vesicles of rats from the various treatment groups on day 18.

Neonatal	ERα		ERβ		AR		PR	
treatment	Stroma	Epithelium	Stroma	Epithelium	Stroma	Epithelium	Stroma	Epithelium
Control	+	–	+++	+++	+++	+++	–	–
DES 10 µg	+++	–	+++	+++	+	–	+++	–
EE	++	–	+++	+++	++	+	++	–
Tamoxifen	++	–	+++	+++	+++	+++	++	–
Bisphenol A	+	–	+++	+++	+++	+++	–	–
Octylphenol	+	–	+++	+++	+++	+++	–	–
Genistein	+	–	+++	+++	+++	+++	–	–
GnRHa	++/+	–	+++	+++	+++	+++	–	–
Flutamide	++	–	+++	+++	++	++	++	–

Results are based on the semiquantification of sections from at least six animals per group (except for tamoxifen, where *n* = 3) and by comparison with sections from control and DES 10 µg–treated animals.

Results

Effect of neonatal treatments on gross morphology of the SVs. In DES (10 µg)-treated animals, the SVs were reduced in size and exhibited a massive increase in the relative proportions of stromal to epithelial tissue when compared with controls (Figure 1, Table 1); this was probably the result of undergrowth of the epithelium, which was very sparse. The noticeable reduction in branching of the SV epithelium when compared with controls (Figures 1–3, Table 1) was one manifestation of this change. Similar but less pronounced changes in SV morphology were also evident in animals treated with DES (1 µg; Figures 1 and 2). Neonatal treatment with 10 µg EE caused changes in SV morphology that were intermediate between those induced by the 10- and 1-µg doses of

DES (not shown, but summarized in Table 1). Tamoxifen treatment induced patchy disturbance of stroma:epithelial proportions, and this treatment and flutamide appeared to cause minor reductions in epithelial branching (Figure 3, summarized in Table 1). None of the other treatments caused any detectable change in stromal:epithelial proportions or branching in the SVs (Table 1, Figure 1).

Immunoexpression of sex steroid receptors in SVs of control rats at day 18. In controls, ARs were immunoexpressed intensely in the nuclei of most epithelial cells and many stromal cells (Figure 2). In contrast, PR imunoexpression was absent from all cells, and immunoexpression of ERα was restricted to a small number of stromal cells (Figure 2). The cellular distribution of ERβ immunoexpression in control rats was comparable to that of AR, and there was no consistent difference in the intensity of immunoexpression in epithelial and stromal cell nuclei (Table 2, Figure 3).

Effect of treatment with DES, EE, or GnRHa on sex steroid receptor immunoexpression in SVs at day 18. Neonatal treatment with DES produced major, dose-dependent changes in the immunoexpression of AR, PR, and ERα (Figure 2, Table 2) but no change in ERβ (Table 2, Figure 3; not all data shown). AR immunoexpression in epithelial cells of the SVs was completely absent in DES (10 µg)-treated animals and stromal expression was reduced markedly in intensity when compared with controls. In the same animals there was widespread induction of PR immunoexpression in stromal but not in epithelial cells and a marked increase in the intensity and/or number of cells immunoexpressing ERα in stromal tissue of the SVs. Neonatal administration of a 1-µg dose of DES induced similar but less marked changes in each of these parameters, whereas administration of 0.1 µg DES was without detectable effect when compared with controls (Figure 2). In contrast to the effect of administering 10 µg DES, neonatal treatment with a GnRH antagonist (GnRHa)—which caused retardation of development of the testis, including Leydig cells, comparable to that evident in DES (10 µg)-treated rats (*11,13,39*)—failed to alter AR or PR immunoexpression compared with controls, though a minor but consistent increase in stromal immunoexpression of ERα was evident (Figure 2, Table 2).

Effect of treatment with weak (environmental) estrogens, with tamoxifen, or with the AR antagonist flutamide on sex steroid receptor immunoexpression in SVs at day 18. Neonatal treatment with genistein, octylphenol, or bisphenol-A had no discernible effect on SV morphology or on sex steroid receptor immunoexpression in SVs when compared with controls. None of the changes evident in DES-treated rats, such as relative increase in stromal tissue or induction of stromal PR immunoexpression and loss of epithelial AR immunoexpression, were evident in any of the rats treated with these weak estrogens at a high dose (Figures 1 and 3, Table 2). In contrast, tamoxifen treatment clearly induced PR immunoexpression in stromal cells of the SVs and caused a small increase in stromal ERα immunoexpression, comparable to changes induced by treatment with DES. However, in contrast to the DES treatment, tamoxifen had only minor effects on epithelial AR immunoexpression when compared to the major reduction that was evident in DES (10 and 1 µg doses)-treated rats (Figures 2 and 3, Tables 2 and 3). Neonatal treatment with flutamide also had effects on sex steroid receptor immunoexpression in the SVs (Figure 3, Table 2). It induced stromal PR immunoexpression and slightly but consistently upregulated immunoexpression of ERα in stromal cells, changes consistent with those induced by the two higher doses of DES (Table 2). However, in contrast to DES treatment, flutamide administration caused only a minor reduction in epithelial AR immunoexpression (Figure 3, Table 2). Although it retarded development of the SVs to some extent (they were smaller with less epithelial branching than in controls), flutamide treatment did not detectably alter the relative proportions of stromal and epithelial tissue in the way that DES treatment did (Figure 1, Table 3).

ERβ was immunoexpressed in the nuclei of epithelial and many stromal cells in the SVs. None of the treatments administered, including all three doses of DES, caused any detectable change in the pattern or intensity of immunoexpression of ERβ (Figure 3, Table 2).

Western analysis of sex steroid receptor expression in the SVs at day 18. We used Western analysis to confirm the specificity of the antibodies used for immunolocalization studies and to confirm some of the major changes in sex steroid receptor immunoexpression after some of the treatments.

There was no detectable PR band in protein extracts of SVs from control animals, whereas a band was clearly detectable in SVs from DES (10 µg)- and flutamide-treated rats (Figure 4), both of which showed induction of stromal expression of PR by immunohistochemistry. The apparent M_r of the band detected in the latter two groups comigrated with the band detected in the uterine sample, used as a positive control. The molecular weight of the band detected corresponds to the 110-kDa PR-B form as reported by Estes (*40*).

There was a strong AR band detected in protein extracts of SVs from control and flutamide treated animals, with some indication of a reduction in signal present in the flutamide treatment group (Figure 4). There was no detectable band in the SV protein extract derived from DES treated rats. The

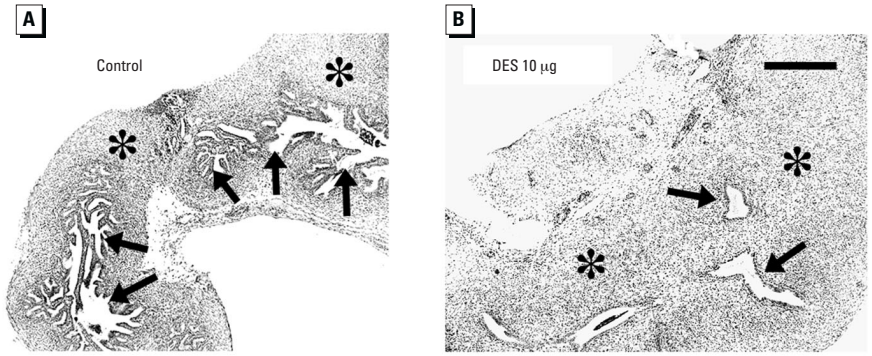

Figure 1. Effect of neonatal hormonal manipulations on the gross structure of the seminal vesicles at day 18 in the rat. Cross-sections show (*A*) the seminal vesicle of a control rat and (*B*) an animal treated neonatally with 10 µg DES. DES treatment greatly reduced the amount of epithelial tissue (arrows) in relation to stromal tissue (asterisks). Reduction in epithelial tissue was also accompanied by a reduction in branching, and the effect of other treatments on this aspect of gross morphology can be judged from Figures 2 and 3. Scale bar = 500 µm.

Table 3. Summary of the association between treatment-induced changes in receptor immunoexpression and the occurrence of major morphologic abnormalities in the seminal vesicles.

Treatment group	Morphologic abnormalities[a]	Receptor immunoexpression change		
		AR	PR	ERα
DES 10 µg	+++	↓↓↓	↑↑↑	↑↑
Tamoxifen	+	↓	↑↑	↑
Flutamide	None[b]	↓	↑↑	↑

[a]Relative increase in stromal tissue and a relative decrease in epithelial tissue. [b]No change in stromal:epithelial proportions but there was retardation of general development.

size of the AR band was approximately 113 kDa, corresponding well with the predicted size of 110 kDa (*41,42*).

The ERα antibody did not recognize recombinant ERβ but detected a band of approximately 75 kDa on Western blots when recombinant ERα was used (Figure 4). Increased expression of ERα in SV extracts from DES (10 μg)- and EE (10 μg)-treated animals was evident, confirming the increased stromal expression of this receptor evident by immunohistochemistry. However, extracts of SVs from flutamide-treated animals showed little apparent change from control values in expression of ERα protein, in contrast to the slightly increased immunoexpression of ERα that was evident in tissue sections (Figure 3).

The ERβ antibody did not recognize recombinant ERα but did detect two bands corresponding to the long (59 kDa) and short (53 kDa) forms of recombinant ERβ. No change in expression of ERβ was evident in SV extracts from any of the treatment groups tested (Figure 4).

Discussion

The present findings confirm and extend our earlier results showing that neonatal estrogen treatment is able to induce major changes in the pattern and intensity of sex steroid receptor expression in a target tissue of the male reproductive system, the SVs. Loss of immunoexpression of AR, especially in epithelial cells of the SVs, was induced by neonatal estrogen treatment coincident with upregulation of ERα (but not ERβ) immunoexpression and the induction of PR immunoexpression in stromal cells. Induction of these changes depended on the dose of DES administered and could be mimicked completely by a synthetic estrogen, EE, and partly by the mixed estrogen agonist/antagonist tamoxifen. Induction of morphologic changes to the SVs, notably relative overgrowth of stromal tissue and undergrowth of epithelial tissue when compared with controls, also coincided with the alterations in immunoexpression of the sex steroid receptors in the DES/EE treatment groups. A novel finding was that neonatal administration of the AR antagonist flutamide was also able to induce some of the changes in receptor expression induced by DES and EE, notably induction of stromal PR expression and a small increase in stromal ERα expression. This was unexpected because these changes coincide with induction of gross morphologic abnormalities in DES-treated rats, whereas neonatal flutamide administration is unable to induce these changes (*9,11*), as

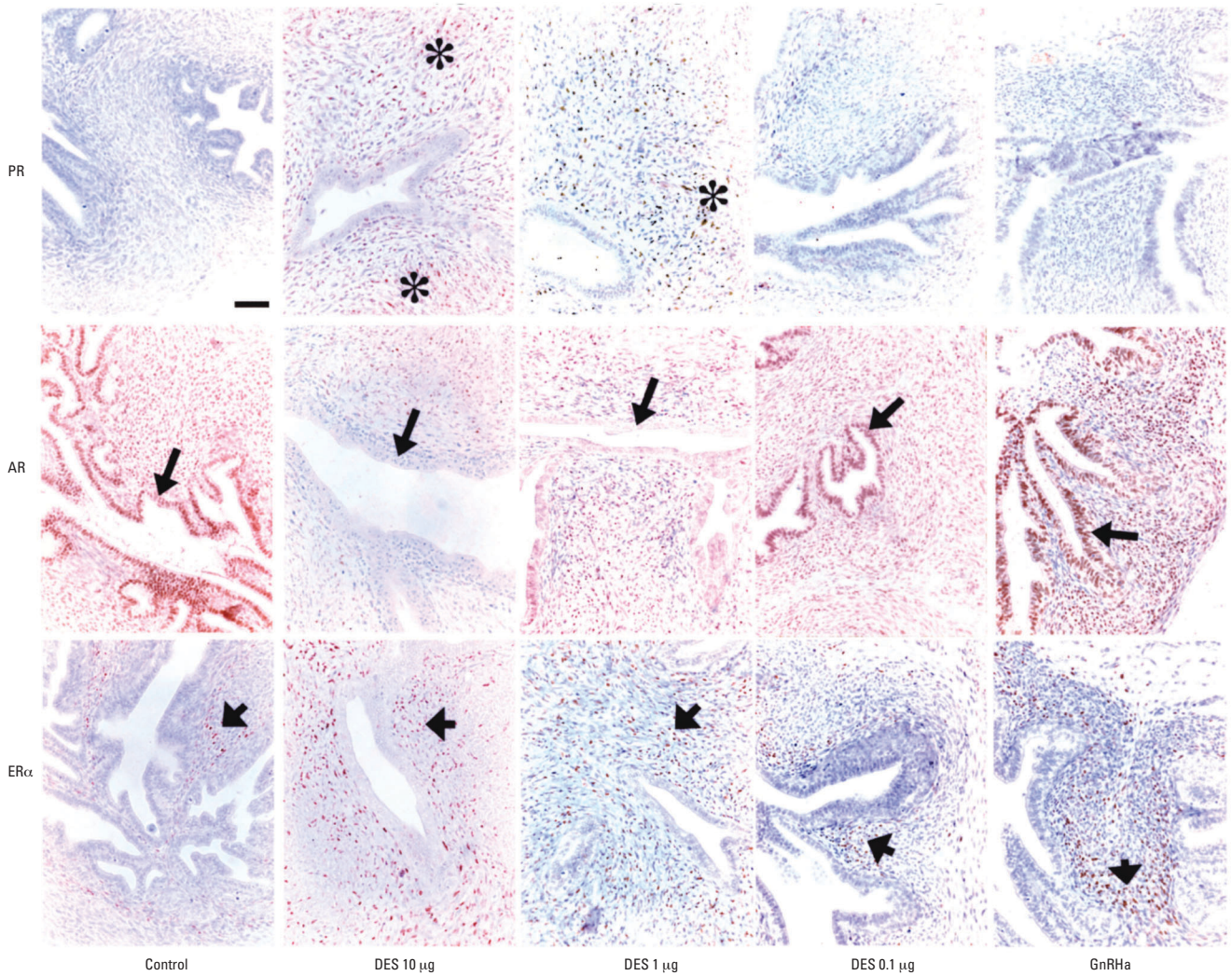

Figure 2. Effect of neonatal treatment with various doses of DES or with GnRHa on immunoexpression (brown staining) of PR, AR, and ERα in the SVs on day 18. Note the complete absence of PR immunoexpression in control SV but its dose-dependent induction in stromal tissue (asterisks) by DES. This induction correlates with loss of epithelial AR immunoexpression (long arrows) and reduction in stromal cell expression of AR when compared with control. Some increase in stromal cell immunoexpression of ERα (short arrows) is also evident in association with induction of PR in DES-treated animals. Note also the dose-dependent inhibition of epithelial branching in SVs from DES-treated animals. SVs from GnRHa-treated rats were indistinguishable from control. Scale bar = 100 μm.

exemplified by its failure to disrupt the stromal:epithelial proportions of the SVs in the present study in the way that DES did. These findings therefore support our suggestion (*11,13*) that some, and possibly all, of the detrimental effects of DES administration on the developing male reproductive tract require disruption of the normal androgen:estrogen balance, such that androgen action is lowered and estrogen action is raised at the same time. In this regard, the present findings showing no effect of neonatal administration of genistein, bisphenol A, or octylphenol at high doses on sex steroid receptor immunoexpression and SV morphology adds to the evidence that only potent estrogens at high doses induce gross abnormalities of the developing male reproductive tract. Induction of changes in the pattern of sex steroid receptors in the developing reproductive system of the male is probably therefore an intrinsic aspect of the mechanisms leading to morphologic abnormalities in neonatally estrogen-treated rats and is perhaps predictive of such changes.

It is well established that perinatal estrogen treatment of rats and mice can induce lifelong changes to the prostate that involve relative overgrowth of stromal tissue and relative undergrowth of epithelial tissue (*9,20,43–46*). Similar changes have also been described for the SVs (*46*), as confirmed in the present studies, and for the epididymis and vas deferens (*10,11*). The mechanisms responsible for these changes have not been defined, though studies of the prostate (*23*) and epididymis/vas deferens (*10,11*) have shown that the changes coincide with loss of expression of the AR. At face value these studies could therefore be interpreted as providing evidence that the morphologic changes induced by perinatal estrogen treatment are simply a consequence of interference with androgen action, an interpretation that fits logically with the key role of androgens in development of the male reproductive system. However, in studies in which we have blocked androgen production (neonatal treatment with a GnRH antagonist) or action (flutamide) in neonatal rats, we have been unable to induce macroscopic changes to the stromal:epithelial tissue ratio in either the epididymis/vas deferens (*11*) or SVs (present study), although both treatments retarded development of the tissues in question (i.e., they were smaller), confirming interference with androgen production/action. Our recent finding that neonatal estrogen treatment was also able to induce stromal cell expression of PR in the epididymis, seminal vesicles, and parts of the prostate (*9*) raised the possibility that this change might play a role, because its induction was also associated with gross changes in the stromal:epithelial ratio. Earlier findings have shown induction of ERs in endometrial (*47*) and epididymal and seminal vesicle tissue (*48*) as a consequence of neonatal estrogen treatment, and our findings suggest that changes in the tissue-specific pattern of expression of ERα may be important in the development of the epididymis and vas deferens (*10*) and similar findings exist for the prostate (*49*). Given these various findings, we surmise that loss of AR expression in combination with induction of PR, and/or with induction of ERs, might be causally linked in triggering changes to the stromal:epithelial ratio. We therefore tested this hypothesis by evaluating expression of each of these receptors in the SVs of rats treated neonatally with potent or weak estrogens, tamoxifen, flutamide, or a GnRH antagonist and relating these to whether or not macroscopic changes to the stromal:epithelial ratio were induced.

Because differences within the SVs in stromal and epithelial expression of AR, PR, and ERα (but not ERβ) were considered likely, our method of choice for these studies was immunocytochemistry, using antibodies for which the specificity had been previously established (see "Methods"). Specificity was

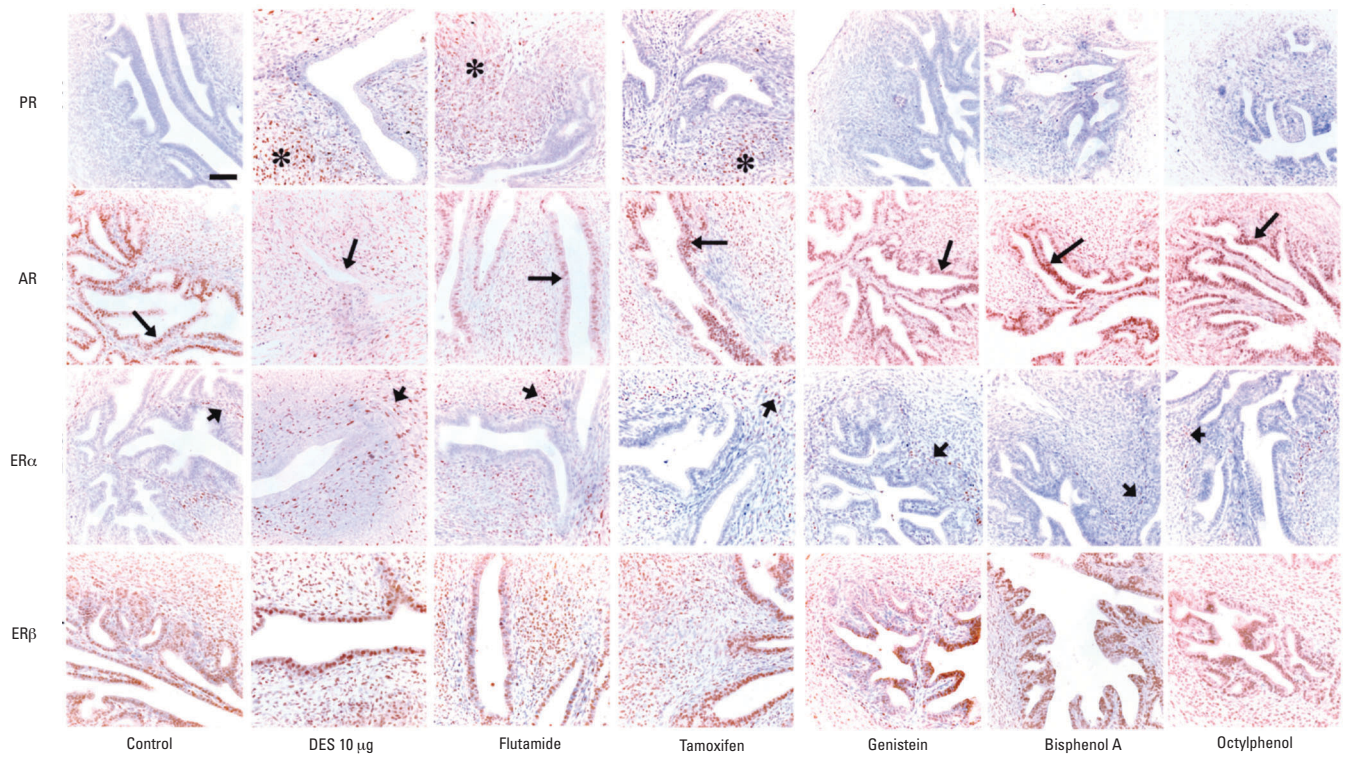

Figure 3. Comparative effects of neonatal treatment with various hormonally active compounds on immunoexpression (brown staining) of PR, AR, ERα and ERβ in the SVs on day 18. Sections of SV from control and DES (10 μg)-treated rats are shown for comparison. Flutamide and tamoxifen, as well as DES, induced stromal immunoexpression of PR (asterisks) and small increases in stromal cell immunoexpression of ERα (small arrows), and there was evidence of reduced epithelial branching in these two groups. However, only DES induced complete loss of epithelial AR immunoexpression (long arrows), although in flutamide-treated animals there was evidence of reduced intensity of epithelial AR expression. Neonatal treatment with genistein, bisphenol A, or octylphenol had no discernible effect on any of the above aspects. Immunoexpression of ERβ was unaffected by any of the treatments. Scale bar = 100 μm.

also confirmed in the present studies, and some of the major treatment-induced changes in receptor immunoexpression on sections were confirmed by Western analysis using the same antibodies. The results obtained confirm our earlier studies (*9*) by showing that neonatal DES treatment dose-dependently induced immunoexpression of PR in stromal tissue of the SVs and demonstrated for the first time that this was associated with loss of AR immunoexpression, especially from epithelial cells, and increased immunoexpression of ERα in stromal cells; these changes were coincident with gross distortion of the stromal:epithelial tissue ratio at the highest DES dose. Similar changes were induced by EE. We therefore tested the effects of tamoxifen treatment because this compound displays a range of effects from complete antagonism to pure estrogen agonism, depending on the concentration, sex of the animal, target organ, and period of use (*50*). Neonatal treatment of male mice with tamoxifen induces various abnormalities to the reproductive tract (*36,51*), and its administration to adult male rats reduced the weight of the SV, ventral prostate, and epididymides (*52,53*). Although tamoxifen treatment was able to induce immunoexpression of PR and to slightly upregulate immunoexpression of ERα in stromal tissue of the SVs in the present studies, it had only minor effects on epithelial immunoexpression of AR and on the stromal:epithelial tissue ratio. The effect of flutamide was tested as it is a pure antiandrogen (*54*) that acts by displacing androgen from and binding to the AR (*55*). Similar to tamoxifen, neonatal treatment with flutamide also induced immunoexpression of PR and induced minor upregulation of the immunoexpression of ERα in stromal tissue but had only minor effects on epithelial immunoexpression of AR and failed to grossly alter the stromal:epithelial tissue ratio. It is emphasized, however, that the SVs from flutamide-treated rats were noticeably smaller than in controls and there was somewhat less epithelial branching.

On the basis of the comparative effects of neonatal treatment with DES, tamoxifen, or flutamide, we conclude that induction of PR expression and upregulation of ERα expression in stromal tissue of the SVs are not sufficient on their own to cause gross abnormalities of SV tissue composition. For the latter to occur, loss of expression of AR, especially in epithelial cells, must also occur. Conversely, treatment-induced loss of AR expression in the reproductive tract of the developing male is associated with induction of stromal PR expression and, at least in some tissues, with altered stromal cell expression of ERα. The one notable exception to the latter generalization is the testis, in which loss of AR expression (*11*) is not accompanied by PR induction (*9*) and is not associated consistently with increased expression of ERα (*11*). The present findings also add to our other results (*9–11*) in demonstrating that expression of ERβ remains unaffected by the different treatments and their associated effects on tissue structure and receptor expression. Although our findings suggest that there could be several pathways via which estrogen treatment induces SV and other reproductive tract abnormalities, loss of AR expression is clearly the dominant factor. How this loss is induced and whether it involves solely ER-mediated or other pathways (e.g., interaction with the AR in some way) remains unknown, and the present findings do not clarify this position.

In our previous studies showing induction of PR expression in male reproductive tissues as a result of neonatal DES treatment, we suggested that this finding might be considered loosely as feminization of the tissues in question, because PR is not normally expressed in the male reproductive system, whereas it is expressed normally in the uterus. However, the present studies show that, in the SVs at least, PR expression can also be induced by treatment with an antiandrogen, and other findings have shown that either castration (*25*) or estrogen treatment in adulthood (*25,26*) can also induce PR expression in the prostate. These various findings are perhaps best reconciled by concluding that disturbance of the androgen:estrogen balance in the male, either by raising estrogen action or by lowering androgen action, is sufficient in some male reproductive tissues (SVs, prostate) to induce PR expression. In this regard there is similar evidence from the female to suggest dual, and opposing, roles of estrogens and androgens in the induction of uterine PR expression (*56*). However, it is notable in the present studies that, whereas flutamide induced PR expression in the SVs, treatment with a GnRH antagonist, which is an effective antiandrogen because of its suppression of androgen production, failed to have any effect. This difference implies that interaction of flutamide with the AR is necessary for PR induction, at least in the developing SVs. It remains unclear why there should be an apparently inherent ability for induction of PR expression in the male reproductive system when it is not normally expressed there. Whether this

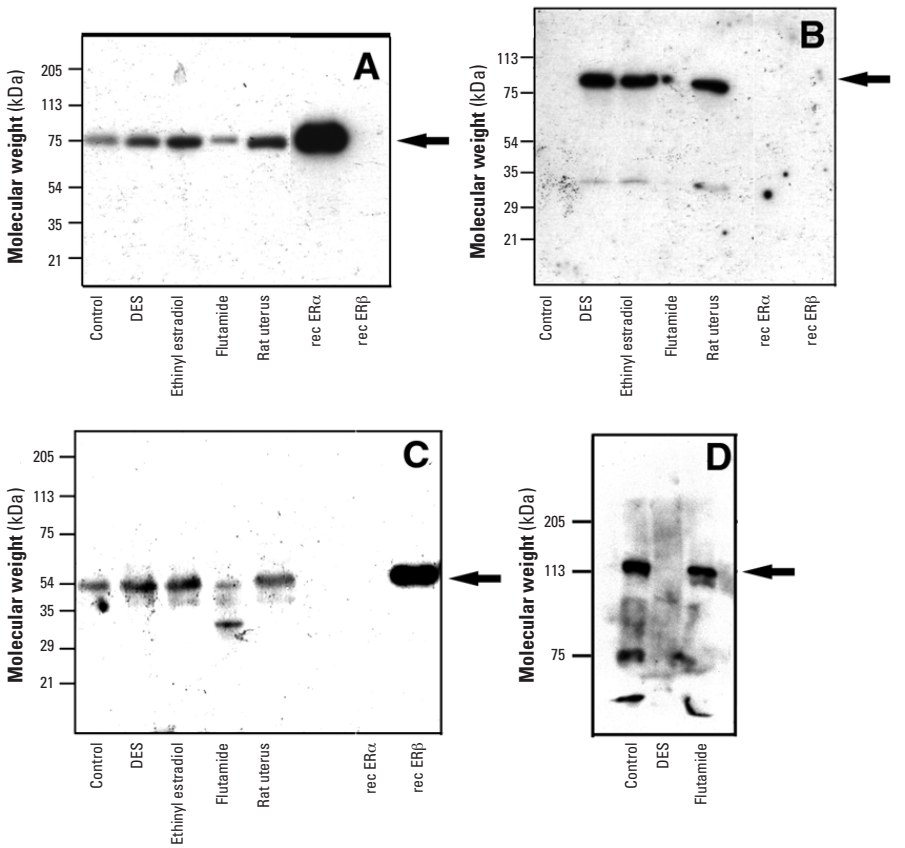

Figure 4. Western blot analysis of (*A*) ERα, (*B*) PR, (*C*) ERβ, and (*D*) AR expression in the SVs on day 18 from selected treatment groups. rec, recombinant. Various positive controls were included, such as recombinant ERα and ERβ or extracts of rat uterus. Arrows show the expected size of the relevant full-length receptor protein. The lower molecular weight bands on the AR blot are breakdown products that probably result from the freeze-thawing that takes place during protein extraction (*11*).

can be interpreted as evidence for lifelong plasticity of the reproductive system is a moot point.

In contrast to the effects of DES, tamoxifen, and flutamide, none of the weak environmental estrogens tested in the present studies (genistein, octylphenol, bisphenol A) was able to affect any of the end points studied, despite the administration of extremely high doses. Based on their weak estrogenicity, this result was perhaps predictable when compared with the dose–response relationship for DES. However, because only a single high dose of each compound was evaluated, we cannot rule out the possibility that lower doses might, paradoxically, be able to induce effects that are then lost at higher doses (*57*). Nevertheless, a logical conclusion from the present studies is that only agents that can grossly suppress AR expression at the same time as increasing or inducing expression of ERα and PR are likely to induce gross developmental abnormalities of the male reproductive system. This conclusion is supported by previous findings (*11*). In this regard, induction of PR expression is certainly a useful guide because its induction was always associated with a change in SV development or gross structure. Moreover, the fact that PR expression is nondetectable in the normal male reproductive system (*9*) means that there is a very clear baseline against which to evaluate any treatment-induced change.

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